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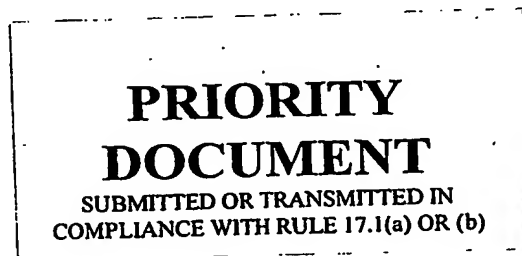PCT/EP03/09116

Bescheinigung   Certificate   Attestation

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Patentanmeldung Nr.   Patent application No.   Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

T-cell epitopes in staphylococcal enterotoxin B

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T-CELL EPITOPES IN STAPHYLOCOCCAL ENTEROTOXIN B

FIELD OF THE INVENTION

5 The present invention relates to polypeptides to be administered especially to humans and in particular for therapeutic use. The polypeptides are modified polypeptides whereby the modification results in a reduced propensity for the polypeptide to elicit an immune response upon administration to the human subject. The invention in particular relates to the modification of staphylococcal

10 enterotoxin B (SEB) to result in SEB proteins that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used *in vivo*. The invention relates furthermore to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified SEB variants with reduced immunogenicity.

15

BACKGROUND OF THE INVENTION

There are many instances whereby the efficacy of a therapeutic protein is limited by an unwanted immune reaction to the therapeutic protein. Several mouse

20 monoclonal antibodies have shown promise as therapies in a number of human disease settings but in certain cases have failed due to the induction of significant degrees of a human anti-murine antibody (HAMA) response [Schroff, R. W. et al (1985) *Cancer Res.* 45: 879-885; Shawler, D.L. et al (1985) *J. Immunol.* 135: 1530-1535]. For monoclonal antibodies, a number of techniques have been

25 developed in attempt to reduce the HAMA response [WO 89/09622; EP 0239400; EP 0438310; WO 91/06667]. These recombinant DNA approaches have generally reduced the mouse genetic information in the final antibody construct whilst increasing the human genetic information in the final construct. Notwithstanding, the resultant "humanized" antibodies have, in several cases, still

30 elicited an immune response in patients [Issacs J.D. (1990) *Sem. Immunol.* 2: 449, 456; Rebello, P.R. et al (1999) *Transplantation* 68: 1417-1420].

Antibodies are not the only class of polypeptide molecule administered as a therapeutic agent against which an immune response may be mounted. Even

proteins of human origin and with the same amino acid sequences as occur within humans can still induce an immune response in humans. Notable examples amongst others include the therapeutic use of granulocyte-macrophage colony stimulating factor [Wadhwa, M. et al (1999) *Clin. Cancer Res.* 5: 1353-

5   1361] and interferon alpha 2 [Russo, D. et al (1996) *Bri. J. Haem.* 94: 300-305; Stein, R. et al (1988) *New Engl. J. Med.* 318: 1409-1413]. In such situations where these human proteins are immunogenic, there is a presumed breakage of immunological tolerance that would otherwise have been operating in these subjects to these proteins.

10

This situation is different where the human protein is being administered as a replacement therapy for example in a genetic disease where there is a constitutional lack of the protein such as can be the case for diseases such as hemophilia A, Christmas disease, Gauchers disease and numerous other

15   examples. In such cases, the therapeutic replacement protein may function immunologically as a foreign molecule from the outset, and where the individuals are able to mount an immune response to the therapeutic, the efficacy of the therapy is likely to be significantly compromised.

20   Irrespective of whether the protein therapeutic is seen by the host immune system as a foreign molecule, or if an existing tolerance to the molecule is overcome, the mechanism of immune reactivity to the protein is the same. Key to the induction of an immune response is the presence within the protein of peptides that can stimulate the activity of T-cells via presentation on MHC class II

25   molecules, so-called "T-cell epitopes". Such T-cell epitopes are commonly defined as any amino acid residue sequence with the ability to bind to MHC Class II molecules. Implicitly, a "T-cell epitope" means an epitope which when bound to MHC molecules can be recognized by a T-cell receptor (TCR), and which can, at least in principle, cause the activation of these T-cells by engaging a TCR to

30   promote a T-cell response.

MHC Class II molecules are a group of highly polymorphic proteins which play a central role in helper T-cell selection and activation. The human leukocyte

antigen group DR (HLA-DR) are the predominant isotype of this group of proteins however, isotypes HLA-DQ and HLA-DP perform similar functions. The present invention is applicable to the detection of T-cell epitopes presented within the context of DR, DP or DQ MHC Class II. In the human population, individuals

5   bear two to four DR alleles, two DQ and two DP alleles. The structure of a number of DR molecules has been solved and these appear as an open-ended peptide binding groove with a number of hydrophobic pockets which engage hydrophobic residues (pocket residues) of the peptide [Brown et al Nature (1993) 364: 33; Stern et al (1994) Nature 368: 215]. Polymorphism identifying the

10 different allotypes of class II molecule contributes to a wide diversity of different binding surfaces for peptides within the peptide binding grove and at the population level ensures maximal flexibility with regard to the ability to recognise foreign proteins and mount an immune response to pathogenic organisms.

15 An immune response to a therapeutic protein proceeds via the MHC class II peptide presentation pathway. Here exogenous proteins are engulfed and processed for presentation in association with MHC class II molecules of the DR, DQ or DP type. MHC Class II molecules are expressed by professional antigen presenting cells (APCs), such as macrophages and dendritic cells amongst

20 others. Engagement of a MHC class II peptide complex by a cognate T-cell receptor on the surface of the T-cell, together with the cross-binding of certain other co-receptors such as the CD4 molecule, can induce an activated state within the T-cell. Activation leads to the release of cytokines further activating other lymphocytes such as B cells to produce antibodies or activating T killer cells

25 as a full cellular immune response.

T-cell epitope identification is the first step to epitope elimination, however there are few clear cases in the art where epitope identification and epitope removal are integrated into a single scheme. Thus WO98/52976 and WO00/34317 teach

30 computational threading approaches to identifying polypeptide sequences with the potential to bind a sub-set of human MHC class II DR allotypes. In these teachings, predicted T-cell epitopes are removed by the use of judicious amino acid substitution within the protein of interest. However with this scheme and

other computationally based procedures for epitope identification [Godkin, A.J. et al (1998) *J. Immunol.* 161: 850-858; Sturniolo, T. et al (1999) *Nat. Biotechnol.* 17: 555-561], peptides predicted to be able to bind MHC class II molecules may not function as T-cell epitopes in all situations, particularly, *in vivo* due to the

5   processing pathways or other phenomena. In addition, the computational approaches to T-cell epitope prediction have in general not been capable of predicting epitopes with DP or DQ restriction.

Equally, *in vitro* methods for measuring the ability of synthetic peptides to bind

10   MHC class II molecules, for example using B-cell lines of defined MHC allotype as a source of MHC class II binding surface and may be applied to MHC class II ligand identification [Marshall K.W. et al. (1994) *J. Immunol.* 152:4946-4956; O'Sullivan et al (1990) *J. Immunol.* 145: 1799-1808; Robadey C. et al (1997) *J. Immunol* 159: 3238-3246]. However, such techniques are not adapted for the

15   screening multiple potential epitopes to a wide diversity of MHC allotypes, nor can they confirm the ability of a binding peptide to function as a T-cell epitope.

Recently techniques exploiting soluble complexes of recombinant MHC molecules in combination with synthetic peptides have come into use [Kern, F. et

20   al (1998) *Nature Medicine* 4:975-978; Kwok, W.W. et al (2001) *TRENDS in Immunol.* 22:583-588]. These reagents and procedures are used to identify the presence of T-cell clones from peripheral blood samples from human or experimental animal subjects that are able to bind particular MHC-peptide complexes and are not adapted for the screening multiple potential epitopes to a

25   wide diversity of MHC allotypes.

Biological assays of T-cell activation remain the best practical option to providing a reading of the ability of a test peptide/protein sequence to evoke an immune response. Examples of this kind of approach include the work of Petra et al using

30   T-cell proliferation assays to the bacterial protein staphylokinase, followed by epitope mapping using synthetic peptides to stimulate T-cell lines [Petra, A.M. et al (2002) *J. Immunol.* 168: 155-161]. Similarly, T-cell proliferation assays using synthetic peptides of the tetanus toxin protein have resulted in definition of

immunodominant epitope regions of the toxin [Reece J.C. et al (1993) *J.*
Immunol. 151: 6175-6184]. WO99/53038 discloses an approach whereby T-cell
epitopes in a test protein may be determined using isolated sub-sets of human
immune cells, promoting their differentiation *in vitro* and culture of the cells in the
5 presence of synthetic peptides of interest and measurement of any induced
proliferation in the cultured T-cells. The same technique is also described by
Stickler et al [Stickler, M.M. et al (2000) *J. Immunotherapy* 23:654-660], where in
both instances the method is applied to the detection of T-cell epitopes within
bacterial subtilisin. Such a technique requires careful application of cell isolation
10 techniques and cell culture with multiple cytokine supplements to obtain the
desired immune cell sub-sets (dendritic cells, CD4+ and or CD8+ T-cells) and is
not conducive to rapid through-put screening using multiple donor samples.

As depicted above and as consequence thereof, it would be desirable to identify
15 and to remove or at least to reduce T-cell epitopes from a given in principal
therapeutically valuable but originally immunogenic peptide, polypeptide or
protein. One of these potential therapeutically valuable molecules is
staphylococcal enterotoxin B (SEB).

20 The enterotoxins of Staphylococcus *aureus* form a group of serologically distinct
extracellular proteins, designated A, B, C_1, C_2, C_3, D, E and F. These proteins
are recognised as the causative agents of Staphylococcal food poisoning.
One of the therapeutic interests in this class of protein stems from their ability to
function as "superantigens", that is molecules able to stimulate the activity of
25 human T-cells. Their therapeutic potential has been tested in a number of clinical
trials where these molecules have been linked to antibodies to provide cell
specific targeting and the superantigen (toxin) has achieved localised T-cell
activation to result in immune mediated suppression of tumour cell growth
[Dohlstein, M et al (1994) *PNAS USA* 91: 8945-8949; Giantonio, B.J. et al (1997)
30 *J. Clin. Oncol.* 15: 1994-2007; Hansson, J. et al (1997) *PNAS USA* 94: 2489-
2494; Alpaugh, K.R. et al (1998) *Clin. Cancer Res.* 4: 1903-1914].
The enterotoxin proteins are of similar molecular weight. Characteristically, they
have a disulfide loop near the middle of the molecule, and are easily soluble in

water and salt solutions. They are relatively resistant to proteolytic enzymes and to heat. The higher level structural similarities between the enterotoxins is in agreement with the clinical picture where all of the enterotoxins seem to produce similar effects of sepsis, hypotension and fever. Amino acid compositions of

5   enterotoxins A, B, C_1, C_2 and E reveal a high content of lysine, aspartic acid and tyrosine. Enterotoxins A and E are similar in methionine, leucine and arginine content, differing in this regard from enterotoxins B, C_1 and C_2.

The present invention is concerned primarily with enterotoxin B and the mature amino acid sequence of SEB (depicted in single-letter code) is as follows:

10   ESQPDPKPDELHKSSKFTGLMENMKVLYDDNHVSAINVKSIDQFLYFDLIYSIKDTKLGNYDNVR
VEFKNKDLADKYKDKYVDVFGANYYYQCYFSKKTNDINSHQTDKRKTCMYGGVTEHNGNQLDKYR
SITVRVFEDGKNLLSFDVQTNKKKVTAQELDYLTRHYLVKNKKLYEFNNSPYETGYIKFIENENS
FWYDMMPAPGDKFDQSKYLMMYNDNKMVDSKDVKIEVYLTTKKK

15   As "superantigens" the Staphylococcal enterotoxins are the most powerful T cell mitogens known eliciting strong polyclonal proliferation at concentrations 10^3 lower than such conventional T cell mitogens as phytohemagglutinin. SEA is the most potent T cell mitogen, stimulating DNA synthesis at concentrations of 10^{-13} to 10^{-16} M in the human system. All stimulate a large proportion human CD4+ and

20   CD8+ T cells. Activity of these mitogens is tightly restricted by the MHC class II antigens. It is proposed that the staphylococcal enterotoxins, and the other superantigen toxins (e.g. streptococcal pyrogenic exotoxins, exfoliative toxins and a product of mycoplasma arthritidis) bind directly to the T cell receptor and to MHC class II. These two structures are brought into contact, thus stimulating T

25   cell activation via the V_β region of the T cell receptor mimicking strong alloreactive response.

Many toxins have binding affinities for MHC class II molecules which are involved in stimulating T cells, SEB has a Kd for human class II of about 10^{-6} M. SEA and SEB probably bind to the same site on class II because they cross compete for

30   binding. The toxins stimulate T cells through V_β binding,T cell receptors for antigenic peptides bound to MHC proteins are made up of 5 clonally variable components V_α, J_α., V_β, D_β.and J_β. Recognition of most conventional antigenic peptides bound to MHC proteins involve contributions from all the variable

components of the T cell receptor. In contrast, the toxins stimulate T cells almost exclusively via the V_β region of the T cell receptor. Bacterial toxins and other superantigens do not bind to T cell receptors at those regions involved in binding to conventional antigenic peptides plus MHC. The

5 superantigens engage V_β on an exposed face of V_β or a region predicted to be a β-pleated sheet and exposed on the side of the T cell receptor. Such a model suggests that toxins act as clamps engaging the sides of class II and V_β bringing into close proximity the surfaces of the T cell receptor and MHC that would contact each other during T cell recognition of conventional antigens bound in the

10 groove of MHC.

It is a particular objective of the present invention to provide modified SEB proteins in which the immune characteristic is modified by means of reduced numbers of potential T-cell epitopes. This immune characteristic is distinct from the functional capability of the whole protein molecule to act as an inducer of T-

15 cell activity via MHC-TCR cross-linking. Rather it is an objective of the present invention to provide for SEB molecules with a retained superantigen activity but a reduced ability to induce an immune response, particularly a T-cell mediated neutralising antibody response. Such a response to the molecule can be expected on its systemic administration for example in fusion with (recombinant

20 or chemical linkage) a targeting moiety for the purpose of anti-tumour therapy or other disease therapy where the immune mediated destruction of a target cell population is a desired therapeutic outcome.

Others have provided modified SEB molecules [US,6,126,945; Pulaski B.A. et al

25 (2000) *Cancer Res.* 60: 2710-2715] but these teachings do not address the importance of T cell epitopes to the immunogenic properties of the protein nor have been conceived to directly influence said properties in a specific and controlled way according to the scheme of the present invention.

30 It is highly desired to provide SEB with reduced or absent potential to induce an immune response in the human subject.

SUMMARY AND DESCRIPTION OF THE INVENTION

The present invention provides for modified forms of SEB, in which the immune characteristic is modified by means of reduced or removed numbers of potential T-cell epitopes.

5

The invention discloses sequences identified within the SEB primary sequence that are potential T-cell epitopes by virtue of MHC class II binding potential. This disclosure specifically pertains the mature SEB protein of 239 amino acid residues.

10

The present invention discloses the major regions of the SEB primary sequence that are immunogenic in man and thereby provides the critical information required to conduct modification to the sequences to eliminate or reduce the immunogenic effectiveness of these sites.

15

In one embodiment, synthetic peptides comprising the immunogenic regions can be provided in pharmaceutical composition for the purpose of promoting a tolerogenic response to the whole molecule.

20   In a further embodiment SEB molecules modified within the epitope regions herein disclosed can be used in pharmaceutical compositions.

In summary the invention relates to the following issues:
- using a panel of synthetic peptides in a naïve T-cell assay to map the
25   immunogenic region(s) of human SEB;
- using a panel of SEB protein variants in a naïve T-cell assay to select variants displaying minimal immunogenicity *in vitro*;
- using a panel of synthetic peptide variants in a naïve T-cell assay to select peptide sequences displaying minimal immunogenicity *in vitro*;
30 - using biological assays of T-cell stimulation to select a protein variant which exhibits a stimulation index of less than 2.0 and preferably less than 1.8 in a naïve T-cell assay;

- construction of a T-cell epitope map of SEB protein using PBMC isolated from healthy donors and a screening method involving the steps comprising:
  i) antigen priming *in vitro* using synthetic peptide or whole protein immunogen for a culture period of up to 7 days; ii) addition of IL-2 and culture for up to 3 days; iii) addition of primed T cells to autologous irradiated PBMC and re-challenge with antigen for a further culture period of 4 days and iv) measurement of proliferation index by any suitable method;

- SEB derived peptide sequences able to evoke a stimulation index of greater than 1.8 and preferably greater than 2.0 in a naïve T-cell assay and selected from any therapeutic protein;

- SEB derived peptide sequences selected from any therapeutic protein having a stimulation index of greater than 1.8 and preferably greater than 2.0 in a naïve T-cell assay wherein the peptide is modified to a minimum extent and tested in the naïve T-cell assay and found to have a stimulation index of less than 2.0;

- SEB derived peptide sequences sharing 100% amino acid identity with the wild-type protein sequence and able to evoke a stimulation index of 1.8 or greater and preferably greater than 2.0 in a T-cell assay;

- an accordingly specified SEB peptide sequence modified to contain less than 100% amino acid identity with the wild-type protein sequence and evoking a stimulation index of less than 2.0 when tested in a T-cell assay;

- an SEB molecule containing a modified peptide sequence which when individually tested evokes a stimulation index of less than 2.0 in a T-cell assay;

- an SEB molecule containing modifications such that when tested in a T-cell assay evokes a reduced stimulation index in comparision to a non modified protein molecule;

- an SEB molecule in which the immunogenic regions have been mapped using a T-cell assay and then modified such that upon re-testing in a T-cell assay the modified protein evokes a stimulation index smaller than the parental (non-modified) molecule and most preferably less than 2.0;

- a modified molecule having the biological activity of SEB and being substantially non-immunogenic or less immunogenic than any non-modified molecule having the same biological activity when used *in vivo*;

5
- an accordingly specified SEB molecule, wherein said loss of immunogenicity is achieved by removing one or more T-cell epitopes derived from the originally non-modified molecule;

- an accordingly specified SEB molecule, wherein said loss of immunogenicity is achieved by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule;

10
- an accordingly specified SEB molecule, wherein one T-cell epitope is removed;

- an accordingly specified SEB molecule, wherein said originally present T-cell epitopes are MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on class II;·

15
- an accordingly specified molecule, wherein 1 – 9 amino acid residues, preferably one amino acid residue in any of the originally present T-cell epitopes are altered;

- an accordingly specified molecule, wherein the alteration of the amino acid residues is substitution, addition or deletion of originally present amino acid(s)
20
residue(s) by other amino acid residue(s) at specific position(s);

- an accordingly specified molecule, wherein, if necessary, additionally further alteration usually by substitution, addition or deletion of specific amino acid(s) is conducted to restore biological activity of said molecule;

- an accordingly specified molecule wherein alteration is conducted at one or
25
more residues from the string of contiguous residues of sequence (a) KFTGLMENMKVLYDDNHVSAI and / or of sequence; (b) QFLYFDLIYSIKDTKLGNYDNVRV and / or of sequence; (c) NKDLADKYKDKYVDVFGANYYYQCYFSKKTNDI and / or of sequence (d) AQELDYLTRHYLVKN wherein said sequences are derived from the SEB wild-
30
type sequence;

- an accordingly specified molecule where in addition to alteration conducted within one or more of the sequences (a), (b), (c) or (d) above, alteration is

conducted within any or all of the peptide sequences (i) – (vi) specified herein where peptide sequence

(i)	PDPKPDELHKSSKFTGLM;
(ii)	YDDNHVSAINVKSIDQFL;
(iii)	YDNVRVEFKNKDLADKYK;
(iv)	TCMYGGVTEHNGNQL;
(v)	VKNKKLYEFNNSPYETGY and
(vi)	NKMVDSKDVKIEVYL;

- a peptide molecule comprising 13–15 consecutive residues from any of sequences (a), (b), (c) or (d) above;
- a peptide molecule comprising at least 9 consecutive residues from any of the sequences (a), (b), (c) or (d) above;
- a peptide molecule comprising 13–15 consecutive residues from any of sequences (i) – (vi) above;
- a peptide molecule comprising at least 9 consecutive residues from any of the sequences (i) – (vi) above;
- a peptide molecule of above sharing greater than 90% amino acid identity with any of the peptide sequences derived from (a), (b), (c) or (d) above;
- a peptide molecule of above sharing greater than 80% amino acid identity with any of the peptide sequences derived from (a), (b), (c) or (d) above;
- peptide sequences as above able to bind MHC class II;
- an accordingly specified SEB molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (a) above;
- an accordingly specified SEB molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (b) above;
- an accordingly specified SEB molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (c) above;
- an accordingly specified SEB molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (d) above;

- an accordingly specified SEB molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequences (a), (b), (c) or (d) above;

- a pharmaceutical composition comprising any of the peptides or modified peptides of above having the activity of binding to MHC class II

- a DNA sequence or molecule which codes for any of said specified modified molecules as defined above and below;

- a pharmaceutical composition comprising a modified molecule having the biological activity of SEB;

- a pharmaceutical composition as defined above and / or in the claims, optionally together with a pharmaceutically acceptable carrier, diluent or excipient;

- a method for manufacturing a modified molecule having the biological activity of SEB as defined in any of the claims of the above-cited claims comprising the following steps: (i) determining the amino acid sequence of the polypeptide or part thereof; (ii) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iii) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iv) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties; and (v) optionally repeating steps (ii) – (iv);

- an accordingly specified method, wherein step (iii) is carried out by substitution, addition or deletion of 1 – 9 amino acid residues in any of the originally present T-cell epitopes;

- an accordingly specified method, wherein the alteration is made with reference to an homologous protein sequence and / or *in silico* modelling techniques;

- a peptide sequence consisting of at least 9 consecutive amino acid residues of a 13mer T-cell epitope peptide as specified above and its use for the

manufacture of SEB having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of SEB when used *in vivo*;

5   The term "T-cell epitope" means according to the understanding of this invention an amino acid sequence which is able to bind MHC class II, able to stimulate T-cells and / or also to bind (without necessarily measurably activating) T-cells in complex with MHC class II.

The term "peptide" as used herein and in the appended claims, is a compound
10 that includes two or more amino acids. The amino acids are linked together by a peptide bond (defined herein below). There are 20 different naturally occurring amino acids involved in the biological production of peptides, and any number of them may be linked in any order to form a peptide chain or ring. The naturally occurring amino acids employed in the biological production of peptides all have
15 the L-configuration. Synthetic peptides can be prepared employing conventional synthetic methods, utilizing L-amino acids, D-amino acids, or various combinations of amino acids of the two different configurations. Some peptides contain only a few amino acid units. Short peptides, e.g., having less than ten amino acid units, are sometimes referred to as "oligopeptides". Other peptides
20 contain a large number of amino acid residues, e.g. up to 100 or more, and are referred to as "polypeptides". By convention, a "polypeptide" may be considered as any peptide chain containing three or more amino acids, whereas a "oligopeptide" is usually considered as a particular type of "short" polypeptide. Thus, as used herein, it is understood that any reference to a "polypeptide" also
25 includes an oligopeptide. Further, any reference to a "peptide" includes polypeptides, oligopeptides, and proteins. Each different arrangement of amino acids forms different polypeptides or proteins. The number of polypeptides–and hence the number of different proteins–that can be formed is practically unlimited. "Alpha carbon (Cα)" is the carbon atom of the carbon-hydrogen (CH) component
30 that is in the peptide chain. A "side chain" is a pendant group to Cα that can comprise a simple or complex group or moiety, having physical dimensions that can vary significantly compared to the dimensions of the peptide.

The invention may be applied to any SEB species of molecule with substantially the same primary amino acid sequences as those disclosed herein and would include therefore SEB molecules derived by genetic engineering means or other processes and may contain more or less than 239 amino acid residues.

5 Streptococcal enterotoxins A, C, C_1, C_2, D, E and F also other related toxins from different microbial sources have in common many of the peptide sequences of the present disclosure and have in common many peptide sequences with substantially the same sequence as those of the disclosed listing. Such protein sequences equally therefore fall under the scope of the present invention.

10

The invention is conceived to overcome the practical reality that soluble proteins introduced with therapeutic intent in man trigger an immune response resulting in development of host antibodies that bind to the soluble protein. The present invention seeks to address this by providing SEB proteins with altered propensity

15 to elicit an immune response on administration to the human host. According to the methods described herein, the inventors have discovered the regions of the SEB molecule comprising the critical T-cell epitopes driving the immune responses to this protein.

20 The general method of the present invention leading to the modified SEB comprises the following steps:

(a) determining the amino acid sequence of the polypeptide or part thereof;

(b) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of

25 the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays;

(c) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the

30 peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays. Such sequence variants are created in such a way to avoid creation of new potential T-cell epitopes by the sequence variations unless such new

potential T-cell epitopes are, in turn, modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope; and

(d) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable

5   properties according to well known recombinant techniques.

The identification of potential T-cell epitopes according to step (b) can be carried out according to methods describes previously in the art. Suitable methods are disclosed in WO 98/59244; WO 98/52976; WO 00/34317 and may preferably be

10   used to identify binding propensity of SEB-derived peptides to an MHC class II molecule. In addition, where multiple potential epitopes are identified and in particular where a number of peptide sequences are found to be able to stimulate T-cells in a biological assay, cognisance may also be made of the structural features of the protein in relation to its propensity to evoke an immune response

15   via the MHC class II presentation pathway. For example where the crystal structure of the protein of interest is known the crystallographic B-factor score may be analysed for evidence of structural disorder within the protein, a parameter suggested to correlate with the proximity to the biologically relevant immunodominant peptide epitopes [Dai G. et al (2001) *J. Biological Chem.* 276:

20   41913-41920]. Such an analysis when conducted on the SEB crystal structure suggests the SEB sequence regions defined herein as (a), (b) and (c) are likely to be immunodominant to sequence region (d). Similarly, for the additional peptides (i) – (iv) defined herein, the B-factor analysis indicates that peptides (i) – (iv) are more likely to be biologically relevant immunodominant sequences then peptides

25   (v) and (vi) which could be cryptic epitopes detected using a naive human T-cell assay.

In practice a number of variant SEB proteins will be produced and tested for the desired immune and functional characteristic. The variant proteins will most

30   preferably be produced by recombinant DNA techniques although other procedures including chemical synthesis of SEB fragments may be contemplated.

The invention relates to SEB analogues in which substitutions of at least one amino acid residue have been made at positions resulting in a substantial reduction in activity of or elimination of one or more potential T-cell epitopes from the protein. It is most preferred to provide SEB molecules in which amino acid

5 modification (e.g. a substitution) is conducted within the most immunogenic regions of the parent molecule. The major preferred embodiments of the present invention comprise SEB molecules for which any of the MHC class II ligands are altered such as to eliminate binding or otherwise reduce the numbers of MHC allotypes to which the peptide can bind. The inventors herein have discovered

10 immunogenic regions of the SEB molecule in man comprising the consecutive amino acid sequences;

  (a) KFTGLMENMKVLYDDNHVSAI;

  (b) QFLYFDLIYSIKDTKLGNYDNVRV;

  (c) NKDLADKYKDKYVDVFGANYYYQCYFSKKTNDI

15 (d) AQELDYLTRHYLVKN.

Six additional peptide sequences may also be considered for alteration under the scheme of the present, such additional peptides having now been demonstrated to be capable of functioning as MHC class II ligands and stimulating T-cells according to recognised methods. The additional peptide sequences are:

20 i) PDPKPDELHKSSKFTGLM

  ii) YDDNHVSAINVKSIDQFL

  iii) YDNVRVEFKNKDLADKYK

  iv) TCMYGGVTEHNGNQL

  v) VKNKKLYEFNNSPYETGY

25 vi) NKMVDSKDVKIEVYL

For the elimination of T-cell epitopes, amino acid substitutions are preferably made at appropriate points within the peptide sequence predicted to achieve substantial reduction or elimination of the activity of the T-cell epitope. In practice

30 an appropriate point will preferably equate to an amino acid residue binding within one of the pockets provided within the MHC class II binding groove.

It is most preferred to alter binding within the first pocket of the cleft at the so-called P1 or P1 anchor position of the peptide. The quality of binding interaction between the P1 anchor residue of the peptide and the first pocket of the MHC class II binding groove is recognized as being a major determinant of overall

5   binding affinity for the whole peptide. An appropriate substitution at this position of the peptide will be for a residue less readily accommodated within the pocket, for example, substitution to a more hydrophilic residue. Amino acid residues in the peptide at positions equating to binding within other pocket regions within the MHC binding cleft are also considered and fall under the scope of the present.

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It is understood that single amino acid substitutions within a given potential T-cell epitope are the most preferred route by which the epitope may be eliminated. Combinations of substitution within a single epitope may be contemplated and for example can be particularly appropriate where individually defined epitopes are in

15   overlap with each other. Moreover, amino acid substitutions either singly within a given epitope or in combination within a single epitope may be made at positions not equating to the "pocket residues" with respect to the MHC class II binding groove, but at any point within the peptide sequence. Substitutions may be made with reference to an homologues structure or structural method produced using *in*

20   *silico* techniques known in the art and may be based on known structural features of the molecule according to this invention. All such substitutions fall within the scope of the present invention.

Amino acid substitutions other than within the peptides identified above may be

25   contemplated particularly when made in combination with substitution(s) made within a listed peptide. For example a change may be contemplated to restore structure or biological activity of the variant molecule. Such compensatory changes and changes to include deletion or addition of particular amino acid residues from the SEB polypeptide resulting in a variant with desired activity and

30   in combination with changes in any of the disclosed peptides fall under the scope of the present.

In as far as this invention relates to modified SEB, compositions containing such modified SEB proteins or fragments of modified SEB proteins and related compositions should be considered within the scope of the invention. In another aspect, the present invention relates to nucleic acids encoding modified SEB

5   entities. In a further aspect the present invention relates to methods for therapeutic treatment of humans using the modified SEB proteins. In this aspect the modified SEB protein may be linked with an antibody molecule or fragment of an antibody molecule. The linkage may be by means of a chemical cross-linker or the SEB-antibody moiety may be produced as a recombinant fusion protein.

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In a further aspect still, the invention relates to methods for therapeutic treatment using pharmaceutical preparations comprising peptide or derivative molecules with sequence identity or part identity with the sequences herein disclosed.

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PATENT CLAIMS:

1. A molecule having the biological activity of staphylococcal enterotoxin B (SEB) containing one or more amino acid modifications such that when tested in a T-cell assay evokes a reduced stimulation index in an individual in vivo in comparision to the non modified protein molecule in the same individual.

2. A SEB molecule of claim 1 containing a modified peptide sequence which when tested in an individual evokes a reduced immunogenicity measured by a stimulation index of less than 2.0 in a T-cell assay.

3. A SEB molecule according to claim 1 or 2, wherein said loss of immunogenicity is achieved by removing one or more T-cell epitopes derived from the originally non-modified molecule.

4. A SEB molecule of claim 3, wherein one T-cell epitope is removed;

5. A SEB molecule of claim 3 or 4, wherein said loss of immunogenicity is achieved by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule.

6. A SEB molecule according to any of the claims 1 to 5, wherein said originally present T-cell epitopes are MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on class II.

7. A SEB molecule of claim 6, wherein 1 – 6 amino acid residues, preferably one amino acid residue in any of the originally present T-cell epitopes are altered.

8. A SEB molecule of claim 7, wherein the alteration of the amino acid residues is substitution, addition or deletion of originally present amino acid(s) residue(s) by other amino acid residue(s) at specific position(s).

9. A SEB molecule of claim 7 or 8, wherein alteration is conducted at one or more residues from the string of contiguous residues of the EPO wild-type sequence sequence

(a) KFTGLMENMKVLYDDNHVSAI;

(b) QFLYFDLIYSIKDTKLGNYDNVRV;

(c) NKDLADKYKDKYVDVFGANYYYQCYFSKKTNDI

(d) AQELDYLTRHYLVKN.

10. An EPO molecule of claim 9, wherein in addition to alteration conducted within one or more of the sequences (a), (b), (c) or (d), alteration is conducted within any or all of the peptide sequences (i) – (iv):

    (i)   PDPKPDELHKSSKFTGLM

    (ii) YDDNHVSAINVKSIDQFL

    (iii) YDNVRVEFKNKDLADKYK

    (iv) TCMYGGVTEHNGNQL

    (v)   VKNKKLYEFNNSPYETGY

    (vi) NKMVDSKDVKIEVYL

11. A T-cell epitope peptide comprising 13–15 consecutive residues from any of sequences (a), (b), (c) or (d) of claim 10.

12. A T-cell epitope peptide of claim 11 comprising at least 9 consecutive residues from any of the sequences (a), (b), (c) or (d).

13. A T-cell epitope peptide of claim 11 or 12 sharing greater than 90% amino acid identity with any of the peptide sequences derived from (a), (b), (c) or (d).

14. A T-cell epitope peptide of claim 11 or 12 sharing greater than 80% amino acid identity with any of the peptide sequences derived from (a), (b), (c) or (d).

15. A T-cell epitope peptide of any of the claims 11 to 14 which is able to bind MHC class II.

16. A SEB derived peptide sequence according to any of the claims 11 to 15 which is able to evoke a stimulation index of greater than 1.8 and preferably greater than 2.0 in a naïve T-cell assay and selected from any therapeutic protein.

17. A peptide deriving from a peptide of claim 16, wherein the peptide is modified to a minimum extent by exchange of one or more amino acid residues having a stimulation index of less than 2.0 in a T-cell assay.

18. A DNA sequence or molecule which codes for any of said SEB molecules and T-cell epitopes as specified in any of the claims 1 to 17.

19. A pharmaceutical composition comprising any of said SEB molecules and T-cell epitopes as specified in any of the claims 1 to 18 and having the activity of binding to MHC class II together with a pharmaceutically acceptable carrier, diluent or excipient.

20. Use of a peptide sequence according to any of the claims 11 to 17 for the manufacture of SEB having substantially no or less immunogenicity in an individual than any non-modified molecule having the biological activity of SEB when used *in vivo* in the same individual.

21. A method for manufacturing a modified molecule having the biological activity of SEB as defined in any of the claims of the above-cited claims comprising the following steps: (i) determining the amino acid sequence of the polypeptide or part thereof; (ii) identifying one or more potential T-cell

epitope peptides within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iii) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iv) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties; and (v) optionally repeating steps (ii) – (iv),

wherein step (ii) is carried out by one or more of the following sub-steps:

(a) using a panel of synthetic peptides in a naïve T-cell assay to map the immunogenic region(s) of SEB;

(b) using a panel of SEB protein variants in a naïve T-cell assay to select variants displaying minimal immunogenicity *in vitro*;

(c) using a panel of synthetic peptide variants in a naïve T-cell assay to select peptide sequences displaying minimal immunogenicity *in vitro*;

(d) using biological assays of T-cell stimulation to select a protein variant which exhibits a stimulation index of less than 2.0 and preferably less than 1.8 in a naïve T-cell assay;

(e) construction of a T-cell epitope map of SEB protein using PBMC isolated from healthy donors and a screening method involving the steps comprising: (1) antigen priming *in vitro* using synthetic peptide or whole protein immunogen for a culture period of up to 7 days; 2) addition of IL-2 and culture for up to 3 days; 3) addition of primed T cells to autologous irradiated PBMC and re-challenge with antigen for a further culture period of 4 days and 4) measurement of proliferation index by any suitable method;

22. The method of claim 21, wherein the alteration is made with reference to an homologous protein sequence and / or *in silico* modelling techniques.

Abstract

The present invention relates to polypeptides to be administered especially to humans and in particular for therapeutic use. The polypeptides are modified polypeptides whereby the modification results in a reduced propensity for the polypeptide to elicit an immune response upon administration to the human subject. The invention in particular relates to the modification of staphylococcal enterotoxin B (SEB) to result in SEB proteins that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used *in vivo*. The invention relates furthermore to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified SEB variants with reduced immunogenicity.